Agarivorans albus gen. nov., sp. nov., a γ -proteobacterium isolated from marine animals

Midori Kurahashi and Akira Yokota

Correspondence

Midori Kurahashi

kuramido@iam.u-tokyo.ac.jp

Institute of Molecular and Cellular Biosciences, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113-0032, Japan

Six bacterial strains were isolated from healthy marine organisms that were collected from the coast of the Kanto area in Japan. Phylogenetic analysis based on 16S rDNA sequence similarity showed that the six isolates formed a separate cluster in the γ -Proteobacteria and were related to the genera *Alteromonas* and *Glaciecola* (<91.6% similarity). The isolates were related closely to each other (DNA–DNA reassociation values of 74–93%). The isolates had a polar flagellum and were Gram-negative, mesophilic, strictly aerobic rods that required salt for growth. Distinct phenotypic features of this group included the ability to hydrolyse agar and white pigmentation of colonies. The DNA G+C content of the isolates was 48–50 mol%. The major quinone was Q-8. Phenotypic characteristics of the isolates differed from those of members of the genera *Alteromonas* and *Glaciecola*. The name *Agarivorans albus* gen. nov., sp. nov. is proposed for the six isolates; the type strain is MKT 106^T (=IAM 14998^T=LMG 21761^T).

INTRODUCTION

The objective of the present study was to shed light on the diversity of bacteria in the intestines of marine creatures, especially Mollusca. In total, 116 bacterial strains were isolated from the intestines of 21 species of marine creatures, including molluscs and protochordata, that were collected at a depth of 15 m in sea water 15 m from the shore.

Partial 16S rDNA sequencing revealed that the isolated strains belonged to the γ -Proteobacteria, α -Proteobacteria and the *Cytophaga*–*Flavobacterium*–*Bacteroides* group. BLAST searches revealed that the complete 16S rDNA sequences of 17 of the 116 isolates had <94% similarity with 16S rDNA sequences that had been deposited in the databases. Of these 17 isolates, six belonged to the *Alteromonas* group and six were related closely to each other, representing a novel lineage within the γ -Proteobacteria. Based on our polyphasic taxonomic study, a novel bacterium, *Agarivorans albus* gen. nov., sp. nov., is described here.

METHODS

Sampling. The strains used in this study were obtained from internal organs of reference marine creatures (Table 1). Marine creatures

were collected by scuba-diving or by wading off the coasts of Izu-Osezaki, Shizuoka Prefecture and Izu-Ohshima Island, Japan, in 1999–2000.

Isolation of bacterial strains. The collected marine creatures were washed several times with sterile sea water. Excised gastro-intestinal tracts and attached internal organs were homogenized and diluted serially to a ratio of 1:10 in sterile sea water. Aliquots (0.1 ml each) of the dilution were spread onto marine agar 2216 (Difco). Plates were then incubated at 23 °C for 1 week. Colonies that appeared on the plates were purified by repeated streaking and were then stored by freezing at –80 °C.

Phenotypic characterization. Phenotypic features of the isolates, i.e. colony morphology, pigment production and agar hydrolysis, were determined by cultivating the isolates on marine agar 2216 at 23 °C. Biochemical tests were carried out by using BIOTEST (Eiken Chemical). Flagellation was observed with a model JEOL 1210 transmission electron microscope after negative staining with phosphotungstic acid.

Analyses of fatty acids and isoprenoid quinones. For analysis of whole-cell fatty acids, cells were grown for 24 h at 23 °C on marine agar and analysed by using the GC-based Microbial Identification system (MIDI). Isoprenoid quinones were extracted with chloroform/methanol (2:1, v/v) and purified by TLC, using a mixture of n-hexane and diethyl ether (85:15, v/v) as the solvent. Ubiquinone fractions were analysed by HPLC, using the method described by Collins & Jones (1981).

Genotypic analysis. DNA was extracted by using the method of Marmur (1961). The G+C content of the DNA was determined by HPLC (Tamaoka & Komagata, 1984). DNA–DNA hybridization was carried out at 45 °C with photobiotin-labelled DNA and microplates (Ezaki *et al.*, 1989).

Phylogenetic analysis. Template DNA for PCR was prepared by the method of Hiraishi (1992). 16S rDNA was amplified by using universal primers, as described previously (Kurahashi *et al.*, 2002).

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The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA sequences of strains MKT 82, 86, 87, 89, 106^T and 112 are AB076557–AB076562, respectively.

Table 1. Isolation sources of *Agarivorans albus* strains and sampling sites

Strain designation	Isolation source	Sampling site
MKT 82 (=IAM 14994)	<i>Onchidium</i> sp. (Mollusca, sea slug)	Izu-Ohshima Island (34° 45' N 139° 21' E)
MKT 86 (=IAM 14995)	<i>Dolabella auricularia</i> (Mollusca, sea slug)	Izu-Ohshima Island (34° 45' N 139° 21' E)
MKT 87 (=IAM 14996)	<i>Dolabella auricularia</i> (Mollusca, sea slug)	Izu-Ohshima Island (34° 45' N 139° 21' E)
MKT 89 (=IAM 14997)	<i>Dolabella auricularia</i> (Mollusca, sea slug)	Izu-Ohshima Island (34° 45' N 139° 21' E)
MKT 106 ^T (=IAM 14998 ^T)	<i>Omphalius pfeifferi pfeifferi</i> (Mollusca, limpet)	Tateyama-Chiba (34° 59' N 139° 52' E)
MKT 112 (=IAM 14999)	Unidentified species (Protochordata, sea squirt)	Izu-Osezaki (35° 5' N 138° 47' E)

PCR products were purified with GFX PCR DNA and a gel band purification kit (Amersham Pharmacia Biotech). Purified PCR products were sequenced directly with a BigDye Terminator Cycle Sequencing Ready Reaction kit (Applied Biosystems).

The almost-complete 16S rDNA sequences determined in this work were compared with sequences in GenBank by using the BLAST algorithm (Altschul *et al.*, 1997). Sequences were aligned with those of reference organisms derived from databases. Alignment and phylogenetic analysis were performed using CLUSTAL_W v.1.8 (Thompson *et al.*, 1994). Phylogenetic trees were constructed, based on the K_{nuc} values determined by the neighbour-joining method (Saitou & Nei, 1987). The topology of the phylogenetic tree was evaluated by the bootstrap resampling method of Felsenstein (1985).

RESULTS

16S rDNA sequences and phylogeny

The region that corresponded to the 16S rDNA could be aligned and was 1244 nt long after removal of ambiguous positions. Among the six isolates, 16S rDNA sequence

similarities were 99.4–100.0 %. Results of sequence alignment of these six strains and related strains from GenBank revealed that the newly determined sequences were related to the γ -Proteobacteria. Members of the genera *Glaciecola* and *Alteromonas* were the closest relatives of the six strains (87.8–90.8 % sequence similarity). The phylogenetic tree suggests that the isolates formed a separate cluster; this was supported by a bootstrap value of 100 % (Fig. 1).

Morphological characteristics

Cells were 1.5–1.9 μ m long and 0.7–0.9 μ m wide with a single polar flagellum. They were Gram-negative rods that were straight or curved in shape (Fig. 2). All six isolates formed smooth, rounded colonies that were white in colour.

Biochemical and physiological characteristics

The six isolates exhibited almost-identical characteristics and all were able to hydrolyse agar. After incubating cultures for at least 1 week on marine agar at 23 °C, it was found that

Fig. 1. Phylogenetic tree based on almost-complete 16S rDNA sequences, comparing *Agarivorans albus* with members of *Alteromonas* and related genera. Numbers at branch-nodes are bootstrap values. Bar, 0.01 nucleotide substitutions per position.

Fig. 2. Transmission electron micrograph of *Agarivorans albus* MKT 89 from growing liquid culture. Bar, 1 μ m.

the agar began to liquefy; after several weeks, the agar was completely liquid. The strains did not grow anaerobically. All strains required NaCl for growth; no growth occurred in media that lacked NaCl or in the presence of 10 % NaCl.

In the BIOTEST system (Table 2), the six isolates exhibited oxidase, catalase and β -galactosidase activities and were able to reduce nitrate. On the other hand, lysine decarboxylase and ornithine decarboxylase activities were not detected. Agar and aesculin were hydrolysed, but hydrolysis of gelatin, arginine and urea was not observed. Fructose was utilized as a carbon source for strains MKT 82, 86, 87, 89 and 112, but not for MKT 106^T.

Chemotaxonomic characteristics

The ubiquinone of the six strains was Q-8. The major fatty acids of the strains grown on marine broth are listed in Table 3. The predominant fatty acid of all six strains was non-polar. The major fatty acids of strains MKT 82, 86, 87, 106^T and 112 were C_{16:0}, C_{16:1} ω 7c, C_{18:1} ω 7c and C_{18:1} ω 6c. Anteiso-C_{17:1} ω 9c was the major fatty acid in MKT 89, but this was not common in the remaining five strains.

DNA G+C content and DNA–DNA hybridization

The DNA G+C contents of the six strains ranged from 48.7 to 49.7 mol%. DNA–DNA relatedness values of each strain with the type strain (MKT 106^T) were 92.4 (MKT 82),

75.8 (MKT 86), 82.7 (MKT 87), 90.2 (MKT 89) and 80.7 (MKT 112) %. Levels of DNA–DNA relatedness among these six strains were all above 70 %.

DISCUSSION

Phenotypic analysis gave results that enabled the isolates to be distinguished from the neighbouring genera *Glaciecola* and *Alteromonas*. The most distinctive characteristic of the isolates was their ability to hydrolyse agar; members of the genus *Alteromonas* hydrolyse gelatin, but not agar, and *Glaciecola pallidula* and *Glaciecola punicea* do not hydrolyse either agar or gelatin. An agar-hydrolysing species, *Glaciecola mesophila*, was reported recently (Romanenko *et al.*, 2003). The novel isolates could also grow well in the absence of agar. They formed distinctive white colonies on marine agar 2216, whereas colonies of *Glaciecola* species ranged from pink–red or pale pink to having no pigment and those of *Alteromonas* species had no pigmentation. The isolates had a DNA G+C content of 49–50 mol%, which was slightly higher than those of species of *Glaciecola* (40–46 mol%) and *Alteromonas* (44–47 mol%) (Bowman *et al.*, 1998). Whole-cell fatty acid profiles of these six isolates were similar to those of species of *Glaciecola* and *Alteromonas*. However, C_{18:1} ω 6c, which was one of the major fatty acids of the novel isolates (4.4–14.6 %), was not detected in species of *Glaciecola* or *Alteromonas* (Bowman *et al.*, 1998). Phylogenetic analysis based on 16S rDNA sequences revealed that the six isolates formed a novel taxon. The genus *Alteromonas*, its closest relative, had 90.8 % 16S rDNA sequence similarity to the novel isolates.

The six isolates, which were isolated from four different species of marine creatures, formed a distinct group, exhibiting 99.4–100.0 % 16S rDNA sequence similarity amongst themselves. Moreover, these strains shared 74.4–93.4 % DNA–DNA reassociation, which is clearly above the level of 70 % that is accepted as the limit for species relatedness (Wayne *et al.*, 1987). These results demonstrate clearly that the six isolates belong to the same species.

One of the four species of marine creatures that were used as isolation sources was a tunicate; tunicates are known as seston-feeders. The other three species of molluscs are known as algae-feeders. One particular characteristic of the isolates was the ability to hydrolyse agar. Thus, the MKT strains are possibly important in the feeding habits of the marine creatures from which they were isolated.

In summary, on the basis of morphological, phenotypic and molecular data, the agar-degrading marine isolates can be classified as a novel genus and species, for which the name *Agarivorans albus* gen. nov., sp. nov. is proposed.

Description of *Agarivorans* gen. nov.

Agarivorans (A.ga.ri.vo'rans. N.L. neut. n. *agarum* agar; L. part. adj. *vorans* devouring, destroying; N.L. adj. *agarivorans* agar-devouring).

Table 2. Phenotypic characteristics that differentiate *Agarivorans albus* from previously described *Alteromonas* and *Glaciecola* species

Taxa: 1, *Agarivorans albus*; 2, *Alteromonas*; 3, *Glaciecola*. +, Positive reaction or growth; –, negative reaction or growth; V, variable reaction; NR, not reported.

Characteristic	1	2*	3†
Colony pigmentation	White	None	Pink–red, pale pink, none
Growth in 10 % NaCl	–	+	–
Temperature preference	Mesophilic	Mesophilic	Psychrophilic or mesophilic
Growth at 37 °C	+	+	–
Hydrolysis of:			
Gelatin	–	+	–
Agar	+	–	V
Aesculin	+	NR	V
Urea	–	–	V
Utilization of:			
Mannitol	+	V	V
Glucose	+	+	V
Sucrose	–	+	V
Xylose	+	+	–
Mannose	–	–	V
Fructose	V	+	V
Maltose	+	+	V
DNA G + C content (mol%)	49–50	44–47	40–46
Major fatty acids	$C_{16:0}$, $C_{16:1\omega7c}$, $C_{18:1\omega7c}$, $C_{18:1\omega6c}$	$C_{16:0}$, $C_{16:1\omega7c}$, $C_{17:1\omega8c}$, $C_{18:1\omega7c}$	$C_{16:0}$, $C_{16:1\omega7c}$, $C_{18:1\omega7c}$
16S rDNA sequence similarity‡	100	91	87–89

*Data from Baumann *et al.* (1984), Bowman *et al.* (1998) and Romanenko *et al.* (2003).

†Data from Bowman *et al.* (1998) and Romanenko *et al.* (2003).

‡Similarity (%) to sequences of MKT strains of *Agarivorans albus*.

Gram-negative, strictly aerobic and agar-hydrolysing. Cells are rod-shaped and approximately $1.5\text{--}1.9 \times 0.7\text{--}0.9\text{ }\mu\text{m}$. Spores are not formed; motile by single polar flagella. No growth occurs without NaCl. Mesophilic. Hydrolysis of

agar is observed. Major quinone is ubiquinone-8. Predominant cellular fatty acids are saturated and monounsaturated, straight-chain fatty acids. The genus is affiliated to the γ -*Proteobacteria* and presently contains one species, *Agarivorans albus*, which is the type species.

Table 3. Major fatty acids (%) of *Agarivorans albus*

Taxa: 1, MKT 106^T; 2, MKT 82; 3, MKT 86; 4, MKT 87; 5, MKT 89; 6, MKT 112. ND, Not detected.

Fatty acid	1	2	3	4	5	6
$C_{12:0}$	6.5	6.1	6.5	7.5	6.8	7.0
iso- $C_{16:0}$	4.4	5.5	8.7	3.5	4.2	4.5
$C_{16:1\omega7c}$	25.5	26.1	21.7	32.2	17.6	25.6
$C_{16:0}$	28.7	23.7	22.4	24.2	17.1	25.4
anteiso- $C_{17:1\omega9c}$	ND	ND	ND	ND	23.4	ND
$C_{18:1\omega7c}$	15.3	13.8	18.1	17.4	8.7	16.3
$C_{18:1\omega6c}$	12.1	14.6	9.0	4.4	9.1	8.1

Description of *Agarivorans albus* sp. nov.

Agarivorans albus (al'bus. L. adj. *albus* white).

In addition to the characteristics that define the genus, the following characteristics are observed. Grows on marine agar 2216 and forms colonies with a smooth surface that are thin, circular in shape and white in colour. Major fatty acids are $C_{16:1\omega7c}$, $C_{16:0}$, $C_{18:1\omega7c}$ and $C_{18:1\omega6c}$. Other characteristics are listed in Table 2. Isolated from gastrointestinal tracts and attached internal organs of Mollusca.

The type strain, MKT 106^T (= IAM 14998^T = LMG 21761^T), was isolated from the marine mollusc *Omphalius pfeifferi* *pfeifferi*. DNA G + C content of the type strain is 49.5 mol%.

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